Supporting Information

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SI Materials and Methods

Yeast Strains, Media, and Genetic Techniques. The *S. cerevisiae* strains used in this work are described in Table S1. Standard techniques (1) were used for strain construction and transformation. The strains CHY107, CHY108, CHY115, CHY116, and CHY169 were produced by extending the endogenous *MGT1* ORF in the *S. cerevisiae* strains JD52, JD55, CHY49, or CHY50 (Table S1) with a sequence encoding 13 head-to-tail repeats of the myc epitope tag (1). This was done using a PCR-mediated gene targeting that used pFA6a-13MYC-TRP1 (2), similarly to a previously described procedure that was used for gene disruption (3). *S. cerevisiae* CHY121, CHY122, CHY169, CHY194, CHY195, and CHY219 were constructed through disruptions of *MGT1* or *UFD4* in JD52, JD55, and CHY108 strains (Table S1), using a PCR-mediated gene disruption that used pFA6a-KanMX6 or pFA6a-TRP1 (2) similarly to a previously described procedure (3). *S. cerevisiae* that were null mutants in specific E2 or E3 genes were either from the Varshavsky laboratory's strain collection, or obtained from Dr. Youming Xie (Wayne State University, Detroit, MI), or purchased from Open Biosystems. *S. cerevisiae* media included YPD (1% yeast extract, 2% peptone, 2% glucose; only most relevant media components are cited); SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose); and synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, plus a drop-out mixture of compounds required by a given auxotrophic strain).

Plasmids and Site-Directed Mutagenesis. The plasmids used in this study are cited in Table S2. The low copy (*CEN*-based) pCH336 plasmid expressed $Mgt1_{f3}$ (C-terminally tagged with $flag_3$) from the native P_{MGT1} promoter. To construct pCH336, the promoter region and the *MGT1* ORF were PCR-amplified from *S. cerevisiae* genomic DNA using OCH576/OCH450 and OCH492/OCH577 primers (Table S3). The resulting DNA fragments was digested with BamHI/HindIII and HindIII/XhoI, respectively, and triply ligated into BamHI/XhoI-cut pRS416 (4). Overlap-extension PCR (5) was used to introduce codons encoding Ser^{151} or Met^{151} instead of wild-type (WT) active-site Cys^{151} into the ORF of *MGT1*. A pair of the above PCR primers (OCH576/OCH577) was used, in conjunction with primers OCH540/OCH541 or OCH553/OCH554 (Table S3), for the cloning of pCH337or pCH338, respectively (Table S2). For example, to construct pCH337, which expressed $Mgt1^{C151S}$, two overlapping DNA fragments with a requisite missense mutation were produced initially, using PCR with the primer pairs OCH576/OCH541 and OCH540/OCH577 (Table S3). These fragments were then denatured and reannealed, so that they formed a partially duplex DNA that encompassed the site of mutation contained 3′ overhangs that enabled another PCR, with the primers OCH576 and OCH577 (Table S3), that yielded both the $MGT1^{C151S}$ ORF and its promoter. DNA fragments produced by this procedure (which encoded either $Mgt1^{C151S}$ or $Mgt1^{C151M}$) were digested with BamHI/XhoI and subcloned into BamHI/XhoI-cut pRS416 vector, yielding, respectively, pCH337 and pCH338 (Table S2). The ubiquitin (Ub) reference (UR) (6) plasmids pCH280 and pCH281 were constructed by inserting the SacII/EcoRI-digested, PCR-amplified *MGT1* ORF (using the primer pair OCH494/OCH495) into SacII/EcoRI-cut $pMET416_FUPRCUP9_{NSF}$ (7). To construct pCH285, which expressed GST-Mgt1-His_{10}, the *MGT1-His$_{10}$* ORF, encoding also the C-terminal His_{10} tag, was produced by PCR and the primer pair OCH492/OCH498 (Table S3). The resulting DNA fragment was cloned into BamHI/XhoI-cut pGEX-4T3 (GE Healthcare). Construction details for other plasmids (Table S2) are available upon request. All of the final plasmid constructs were verified by DNA sequencing.

Preparation of Cell Extracts for Immunoblot Analysis. Yeast extracts were prepared using a modification of Kushnirov's method (8). After yeast cells were grown to exponential phase in selective medium, 1 mL of a culture with A_{600} of 1 was centrifuged for 30 sec at 11,200*g*. Cells were resuspended in 1 mL of 0.2 M NaOH, and incubated for 20 min on ice or for 5 min at room temperature, followed by centrifugation for 30 sec at 11,200*g*. The pelleted cells were resuspended in 50 μL of HU buffer (8 M urea, 5% SDS, 1 mM EDTA, 100 mM DTT, 0.005% bromophenol blue, 0.2 M Tris·HCl, pH 6.8) containing protease inhibitor mixture (Sigma–Aldrich), and heated for 10 min at 70 °C. After centrifugation for 5 min at 11,200*g*, 10 μL of supernatant was subjected to SDS/4–12% NuPAGE (Invitrogen) and immunoblotted with either anti-flag (Stratagene), anti-myc, anti-ha, or anti-tubulin antibodies (Sigma–Aldrich).

In vivo Mgt1 polyubiquitylation assays (Fig. 2*B* and the main text) used the plasmids pUB204 ($Ub^{K48R,G76A}$), pUB223 (His_6-$Ub^{K48R,G76A}$), and pCH277 ($Mgt1_{ha}$) (Table S2). *S. cerevisiae* expressing $Mgt1_{ha}$ and overexpressing either $Ub^{K48R,G76A}$ (lane 1) or His_6-$Ub^{K48R,G76A}$ (lane 2) were grown to A_{600} of ≈0.5 in SD medium with required amino acids containing 0.2 mM $CuSO_4$ (to induce the P_{CUP1} promoter) for 3 h before the addition of MG132 to the final concentration of 50 μM for 30 min, followed by a further incubation in the presence of 68 μM MNNG for 1 h. Cell extracts were subjected to Ni-NTA chromatography under denaturing conditions; the retained proteins were fractionated by SDS/4–12% NuPAGE, followed by IB with anti-ha antibody (Fig. 2*C* and the main text).

GST Fusions and Pulldown Assay, and Purification of GST-Mgt1-His_{10} from *E. coli*. The plasmid pCH285 (Table S2), which encoded GST-Mgt1-His_{10}, was transformed into the *E. coli* BL21-Codon Plus (DE3)-RIL strain (Stratagene). A total of 50 mL of overnight culture of transformed cells was inoculated into 1 L of LB medium containing 0.1 mg/ml ampicilin and 34 μg/ml chloramphenicol, followed by growth at 37 °C for ≈1 h to A_{600} of ≈0.8. The expression of GST-Mgt1-His_{10} was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at 30 °C for 4 h. GST-Mgt1-His_{10}, was purified from cell extracts by affinity chromatography with Ni-NTA resin (Qiagen). Specifically, *E. coli* cells were harvested by centrifugation and frozen at -80 °C. Cell pellets were thawed and resuspended in the Ni-NTA binding buffer (0.3 M NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 50 mM NaH_2PO_4/Na_2HPO_4, pH 8.0) containing chicken egg white lysozyme (1 mg/ml; Sigma–Aldrich). The cells were incubated at 4 °C for 30 min and thereafter disrupted by sonication, 3 times for 1 min each at 1-min intervals, followed by the addition of Nonidet P-40 to the final concentration of 0.1%. After centrifugation at 11,200*g* for 30 min, the supernatant was added to a 2-ml Ni-NTA resin (Qiagen, 50% slurry), and incubated for 1 h at 4 °C. The resin was transferred to a 10-mL column and washed 4 times in 10 mL of washing buffer (0.5 M NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 0.1% Nonidet P-40, 50 mM NaH_2PO_4/Na_2HPO_4, pH 8.0). GST-Mgt1-His_{10} was then eluted with 1-mL samples of the binding buffer containing increasing levels of imidazole (25, 50, 100, 150, 200, 250, 300

mM), followed by overnight dialysis against storage buffer (10% glycerol, 0.15 M NaCl, 10 mM β-mercaptoethanol, 50 mM Hepes, pH 7.5).

GST-pulldown assays with extracts from *S. cerevisiae* containing ^{f}Ubr1 were carried out essentially as described in ref. 9, with a slight modification. Either a GST-fusion protein or GST alone (≈5 μg) was incubated with 10 μL GST-Sepharose (50% slurry) in 50 μL of 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0) for 20 min on ice. The beads were washed once with 0.5 mL of GST-loading buffer (10% glycerol, 1% Nonidet P-40, 0.5 M NaCl, 1 mM EDTA, 50 mM Tris·HCl, pH 8.0) and once with 0.5 mL of GST-binding buffer (10% glycerol, 0.05% Nonidet P-40, 50 mM NaCl, 50 mM Hepes, pH 7.8). Samples of washed beads in 0.1 mL of GST-binding buffer were incubated with 0.16-mL samples of yeast extracts containing ^{f}Ubr1 in the absence or presence of indicated dipeptides, at 1 mM each. The beads were washed 3 times with 0.25 mL of GST-binding buffer containing dipeptides at 1 mM, and the bound proteins were eluted by adding 12.5 μL of 2 × SDS loading buffer and incubating the sample at 95 °C for 5 min, followed by SDS/4–12% NuPAGE and immunoblotting with anti-flag antibody.

Pulse–Chase Assays. Pulse–chase experiments were performed essentially as described (7, 10), with slight modifications. *S. cerevisiae* JD52 (WT), JD55 (*ubr1*Δ), and CHY195 (*ubr1*Δ *ufd4*Δ) (Table S1) that carried the plasmids pCH280, pCH437, pCH438, or pCH439 (Table S2) were grown at 30 °C to A_{600} of ≈1 in 10 mL of SD medium with required amino acids. Cells were pelleted by centrifugation and washed with 0.8 mL of the medium. Cell pellets were gently resuspended in 0.4 mL of the same medium and labeled for 5 or 10 min at 30 °C with 0.16 mCi of ^{35}S-EXPRESS (Perkin-Elmer). Cells were pelleted again and resuspended in 0.4 mL of SD medium containing unlabeled L-methionine (10 mM) and L-cysteine (5 mM), in addition to required amino acids. Samples (0.1 mL) were taken at the indicated time points, followed by preparation of extracts, immunoprecipitation with anti-flag antibody cross-linked to agarose beads (Sigma–Aldrich), SDS/4–12% NuPAGE in Mes buffer, and autoradiography.

Mutation Frequency and Sensitivity of Cells to MNNG Treatment. To measure increases in mutation rate that were caused by MNNG, the JD52 (*UBR1*), JD55 (*ubr1*Δ), CHY121 (*mgt1*Δ) or CHY122 (*mgt1*Δ *ubr1*Δ) *S. cerevisiae* were grown in YPD medium (from 200 cells per ml) for ≈2 days (≈38 h) to A_{600} of ≈5. The cells in YPD were incubated in the absence or presence of 34 μM MNNG for 30 min at 30 °C. Cells in 1 mL of culture (A_{600} of ≈5) were pelleted by centrifugation, then washed once in 1 mL of phosphate buffered saline (PBS), then pelleted again, and resuspended in 1 mL of PBS. To determine the frequency of mutations that conferred canavanine resistance, 0.1-mL samples of cells were spread onto SC-Arg plates containing canavanine (60 μg/ml) and incubated for 3 days at 30 °C. To determine the fraction of viable cells, these cell suspensions were diluted 250,000-fold in PBS, and 0.1-mL samples were spread on YPD medium, followed by incubation for 3 days at 30 °C. To determine whether the C-terminal 13-myc epitope of $Mgt1_{m13}$ affects MNNG sensitivity of cells expressing $Mgt1_{m13}$ instead of WT Mgt1, the corresponding *S. cerevisiae* strains were grown to A_{600} of ≈0.5 in YPD medium, followed by the addition MNNG (to the final concentration of 30 μM) for 3 h. The cultures were 5-fold serially diluted, spotted onto YPD medium, and incubated for 2 days at 30 °C. To determine the fraction of surviving cells after treatments with MNNG, the YPH277 (*UBR1*) or YPH277 HR1 (*ubr1*) *S. cerevisiae* strains (Table S1) were grown in YPD medium at 30 °C to A_{600} of ≈1. Stock MNNG solution (6.8 mM in 50 mM Na-acetate, pH 5.0) was added to 1-ml cell suspensions, to the indicated final concentrations of MNNG. Samples were incubated with vigorous shaking for 1 h at 30 °C, followed by appropriate dilutions in PBS, spreading on YPD plates, incubation for 3 days at 30 °C, and measurements of colony numbers.

In Vivo Ubiquitylation Assay. *S. cerevisiae* JD52 that expressed $Mgt1_{ha}$ (from pCH279) and overexpressed either $Ub^{K48R,G76A}$ (from pUB204) or His_6-$Ub^{K48R,G76A}$ (from pUB223) were grown at 30 °C to A_{600} of ≈0.4 in SC(-Trp, -Ura) medium. Cells were then treated with 0.2 mM $CuSO_4$ (to induce the expression of His_6-$Ub^{K48R,G76A}$) for 2.5 h at 30 °C, and thereafter with 68 μM MNNG for 1 h. Pelleted and washed cells were resuspended and placed in the bead-beater in the denaturing lysis buffer [6 M guanidine hydrochloride, 1 mM PMSF, 0.1 M NaH_2PO_4, 10 mM N-ethylmaleimide, 20 mM Tris·HCl (pH 8.0) and protease inhibitor mixture]. The suspension was centrifuged at 11,200*g* for 20 min, and the supernatant was incubated with Ni-NTA agarose beads (Qiagen) for 2 h at 4 °C. The beads were washed 4 times in denaturing lysis buffer, followed by 4 times in wash buffer (0.5 M NaCl 50 mM Tris, pH 8.0). Bound proteins were eluted in elution buffer (1% SDS, 0.1 M EDTA, 0.1 mM DTT, 0.1 M Tris, pH 6.8) and fractionated by SDS/4–12% NuPAGE, followed by immunoblotting with anti-ha antibody (Sigma–Aldrich).

Expression and Purification of Ufd4, Ubr1, Rad6 and Ubc4. The *S. cerevisiae* JD52 (Table S1) that carried p314CUP1FlagUFD4 (Table S2) and expressed the N-terminally flag-tagged Ufd4 (^{f}Ufd4) was grown at 30 °C to A_{600} of ≈4 in 4-L of SC(-Trp) medium containing 0.2 mM $CuSO_4$. The cells were harvested by centrifugation, washed once with PBS, and frozen in liquid N_2. Cells in a frozen pellet (20 g) were broken using glass beads for 5 × 40 sec at the power setting of 6.5 in FastPrep-24 (MP Biomedicals) in 40 mL of lysis buffer (10% glycerol, 0.5% Nonidet P-40, 0.2 M KCl, 1 mM PMSF, 5 mM β-glycerol phosphate, 50 mM Hepes, pH 7.5) containing protease inhibitor mixture *"for use with fungal and yeast extracts"* (Sigma–Aldrich). The suspension was centrifuged at 11,200*g* for 30 min, and the supernatant was mixed with 2 mL of anti-flag M2 affinity beads (Sigma–Aldrich) at 4 °C for 1 h. The beads were collected by centrifugation in Sorvall RT-600B at 1,000 rpm for 5 min at 4 °C, and were washed, sequentially, with 10 mL of lysis buffer, 10 mL of buffer *A* [10% glycerol, 0.5% Nonidet P-40, 1 M KCl, 1 mM EDTA, 50 mM Hepes (pH 7.5), 5 mM β-mercaptoethanol], and 10 mL of buffer *B* (lysis buffer without Nonidet P-40). ^{f}Ufd4 that was bound by the anti-flag antibody was eluted from the beads with buffer *C* (buffer *B* containing flag peptide at 0.5 mg/ml), and thereafter dialyzed at 4 °C overnight against dialysis buffer (10% glycerol, 0.15 M NaCl, 5 mM β-mercaptoethanol, 50 mM Hepes, pH 7.5).

N-terminally flag-tagged Ubr1 (^{f}Ubr1) was overexpressed in *S. cerevisiae* SC295 (Table S1) and purified by fractionation over anti-flag M2 antibody agarose resin as described in ref. 3. *S. cerevisiae* Rad6 or Ubc4 were overexpressed in the *E. coli* BL21-Codon Plus (DE3)-RIL strain and purified as described previously (7, 9).

In Vitro Ubiquitylation Assay. Purified *S. cerevisiae* Uba1(Ub-activating enzyme, E1) and human Ub were from Boston Biochem. S^{35}-labeled $Mgt1_{f3}$ was expressed in the reticulocyte-based in vitro transcription/translation T_NT T7 Quick for PCR DNA kit (Promega), using a PCR-derived DNA fragment that was produced with pCH437 (Table S2) as a template and the primer pair OCH834/OCH438 (Table S3). A total of 2 μ1 of ^{35}S-labeled Mgt1 was incubated with purified ^{f}Ubr1 (0.2 μM), ^{f}Ufd4 (0.2 μM), Rad6 (1 μM), and/or Ubc4 (1 μM) (see Fig. 4) at 30 °C for 15 min in 20 μL of reaction samples (4 mM ATP, 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM DTT, 50 mM Hepes, pH 7.5)

containing 100 nM Uba1 and 80 μM Ub. The reactions were terminated by adding 8 μL of 4 $\times$ SDS/PAGE loading buffer. Samples of 14 μL were heated at 95 °C for 5 min, followed by SDS/4–12% NuPAGE in Mops buffer, and autoradiography.

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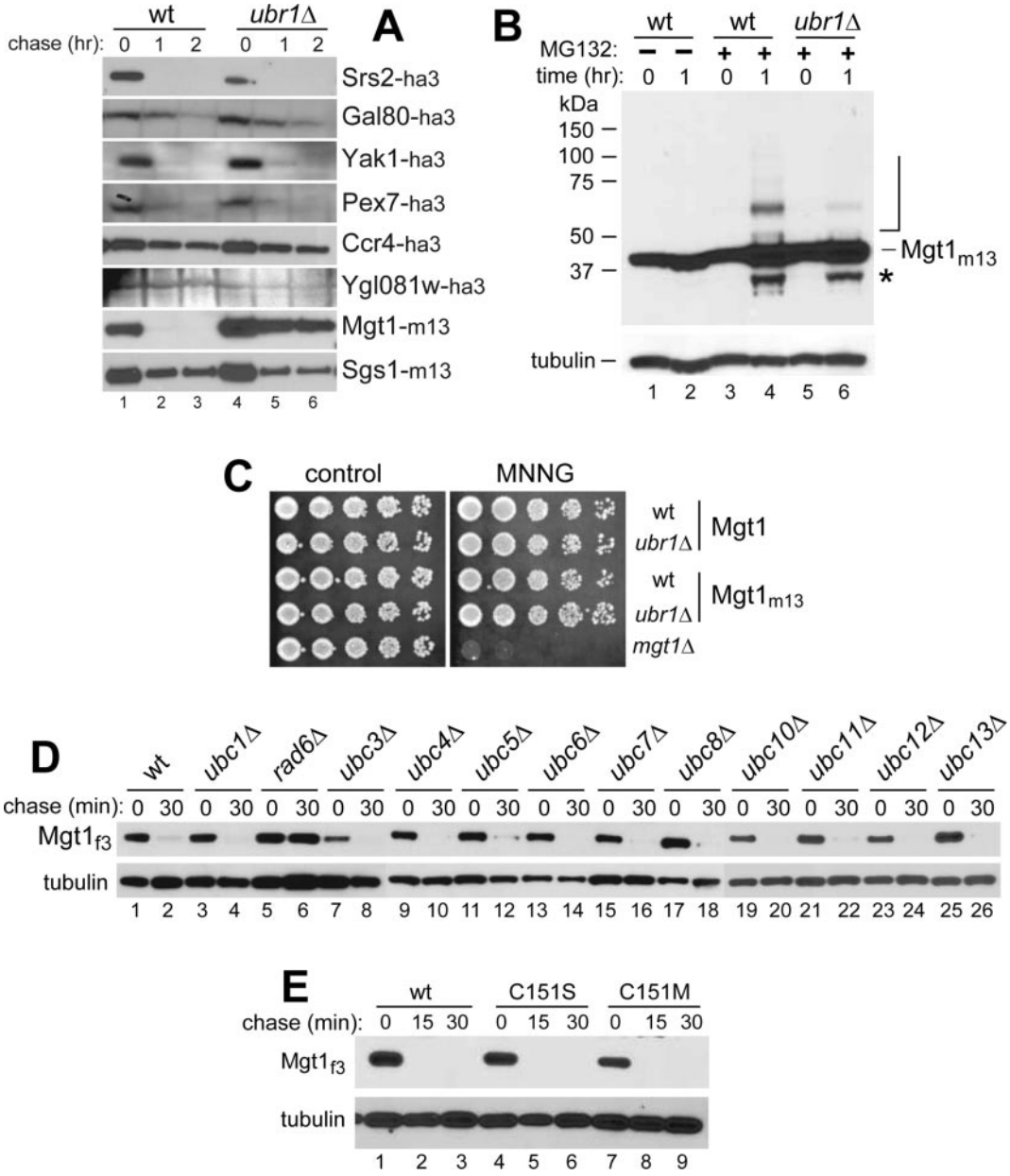

Fig. S1. (*A*) ''Chases'' with cycloheximide to test putative Ubr1-interacting proteins for their degradation by the N-end rule pathway in vivo. The *S. cerevisiae* proteins examined in these experiments were described as proteins that could be coimmunoprecipitated with *S. cerevisiae* Ubr1 in proteomic-scale experiments (1–3). JD52 (WT) and JD55 (*ubr1*Δ) *S. cerevisiae* carrying plasmids (Table S2) that expressed the indicated proteins were grown at 30 °C to A_{600} of ≈1.0, followed by a ''chase'' with cycloheximide for 1 and 2 h, as described in *SI Materials and Methods*, using immunoblotting with anti-ha and anti-myc antibodies. Note that although most of the tested proteins were apparently short-lived in vivo, only Mgt1 was significantly stabilized in the absence of Ubr1. (*B*) Accumulation of ubiquitylated $Mgt1_{m13}$ ($Mgt1_{myc13}$) in the presence of MG132, a proteasome inhibitor. MG132-sensitive *pdr5*Δ *S. cerevisiae* (4) CHY115 cells (*MGT1*$_{myc13}$::*TRP1 pdr5*Δ::*KanMX6*) (Table S1) were grown to A_{600} of ≈1 and incubated further in the presence of either MG132 (50 μM) or dimethyl sulfoxide (an equivalent amount of solvent of the stock solution of MG132) for indicated times at 30 °C. Cell extracts were fractionated by SDS/4–12% NuPAGE and immunoblotted with anti-myc and anti-tubulin antibodies. The (overexposed) band of full-length $Mgt1_{m13}$ and its ubiquitylated derivatives are denoted on the right. Overexposure was used to facilitate the detection of ubiquitylated $Mgt1_{m13}$. An asterisk denotes a (presumed) proteolytic fragment of $Mgt1_{m13}$. (*C*) WT *MGT1* was replaced by an ORF encoding $Mgt1_{m13}$, a derivative C-terminally tagged with the myc_{13} epitope and expressed from the native P_{MGT1} promoter. $Mgt1_{m13}$ was indistinguishable from WT Mgt1 in its ability to protect cells from MNNG. WT or *ubr1*Δ strains expressing either WT Mgt1 or $Mgt1_{m13}$, and a congenic WT strain lacking Mgt1 were grown to A_{600} of ≈0.5 in YPD medium and further grown either in the presence or absence of 30 μM MNNG for 3 h in the same medium. The cultures were serially diluted 5-fold, spotted on YPD plates, and incubated for 2 days at 30 °C. (*D*) The Rad6 E2 enzyme is required for Mgt1 degradation. A set of *S. cerevisiae* null mutants in genes encoding specific E2 (Ub-conjugating) enzymes carried pCH336, which expressed $Mgt1_{f3}$ from the P_{MGT1} promoter. Cells were grown to A_{600} of ≈0.8 at 30 °C, and thereafter incubated at 37 °C for 1 h (to increase the assay's sensitivity; see the main text), followed by a ''chase'' with cycloheximide (50 μg/ml) for 30 min. Cell extracts were fractionated by SDS/4–12% NuPAGE, followed by IB with anti-flag and anti-tubulin antibodies. (*E*) Heat-induced degradation of $Mgt1_{f3}$ is apparently independent of the identity of its residue at position 151. The same strains and procedures as in *A*, except that cells were not treated with MNNG, and were incubated, instead, at 37 °C for 1 h, followed by a ''chase'' with cycloheximide, also at 37 °C.

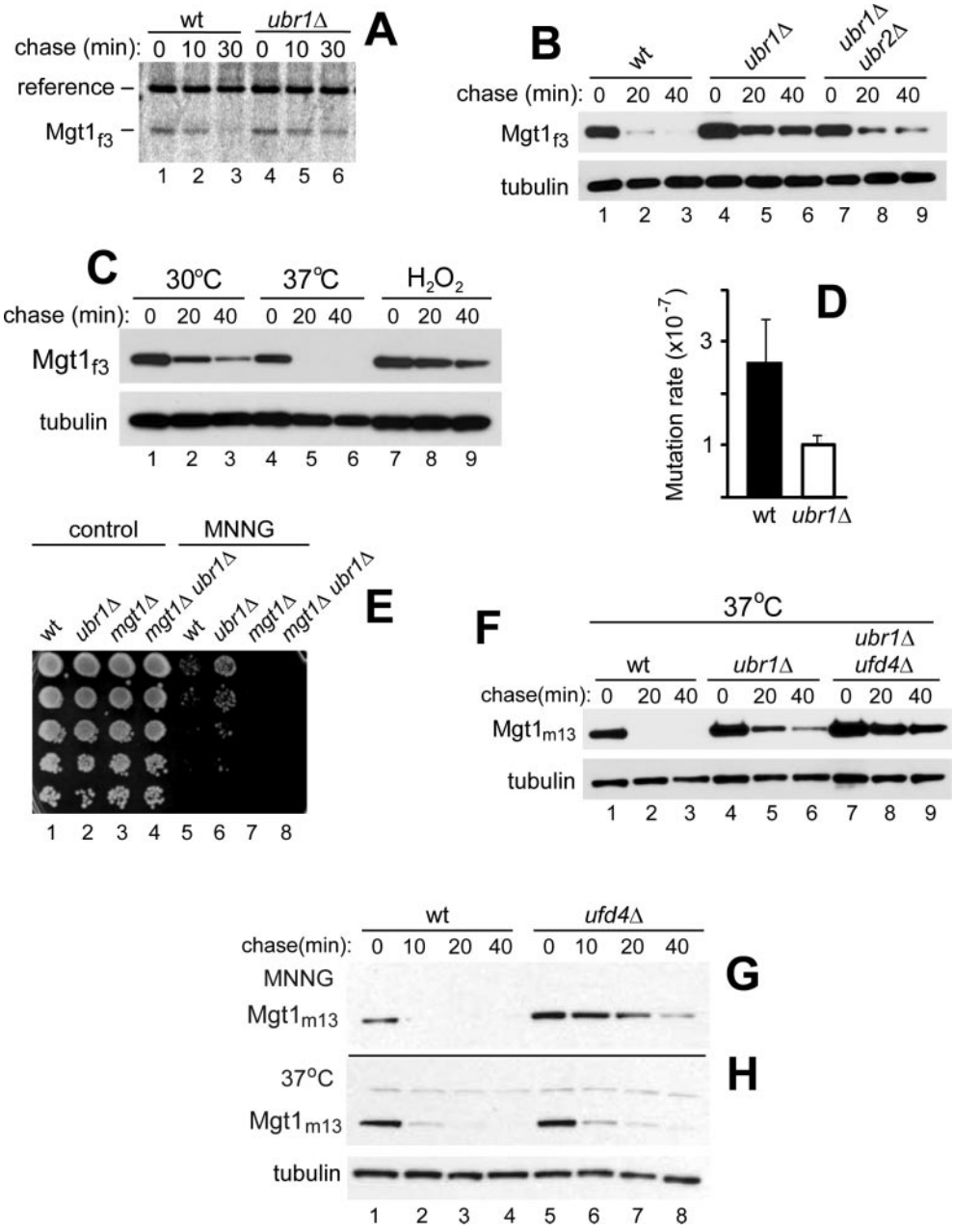

Fig. S2. (*A*) Pulse–chase analysis, using the Ub-reference (UR) technique, of flag-tagged $Mgt1_{f3}$. JD52 (WT) cells (lanes 1–3) and JD55 (*ubr1*Δ) cells (lanes 4–6) expressing ^{f}DHFR-Ub^{K48R}-$Mgt1^{f}$ (pCH281) were grown at 30 °C to A_{600} of ≈1, followed by labeling for 5 min with [^{35}S]methionine/cysteine and a chase (in the presence of cycloheximide) for 0, 10 or 30 min at 30 °C. Cell extracts were precipitated with anti-flag antibody, followed by SDS/4–12% NuPAGE and autoradiography. ''Reference'' on the left denotes the ^{f}DHFR-Ub^{K48R} moiety. (*B*) Cycloheximide ''chase'' assays with JD52 (WT), JD55 (*ubr1*Δ), or double-mutant CHY134 (*ubr1*Δ *ubr2*Δ) *S. cerevisiae* strains expressing $Mgt1_{f3}$. (*C*) WT *S. cerevisiae* expressing $Mgt1_{m13}$ ($Mgt1_{myc13}$) from the P_{MGT1} promoter were grown to A_{600} of ≈0.6, and thereafter preincubated for 1 h at 30 °C (lanes 1–3), or at 37 °C (lanes 4–6), or at 30 °C but in the presence of 1 mM H_2O_2 (lanes 7–9), with the same (preincubation) conditions during cycloheximide ''chases'' for 20 and 40 min. Cell extracts were fractionated by SDS/4–12% NuPAGE, followed by IB with anti-myc and anti-tubulin antibodies. (*D*) Ubr1-lacking *S. cerevisiae* are more resistant to MNNG-induced mutagenesis. JD52 (WT) (filled bar) and JD55 (*ubr1*Δ) (open bar) *S. cerevisiae* were seeded in YPD medium at ≈200 cell/ml, and grown for ≈38 h to A_{600} of ≈5. Cell suspensions were incubated in the presence of 34 μM MNNG for 30 min at 30 °C, then diluted with PBS and spread onto either YPD plates or arginine-lacking SC(-Arg) plates containing canavanine (60 μg/ml), to select for canavanine-resistant cells. Plates were incubated for 3 days at 30 °C, and the resulting colonies were counted. (*E*) Cells of indicated genotypes (Table S1) were grown to A_{600} of ≈0.6 in YPD medium at 30 °C, and were further incubated in the presence of 0.1 mM MNNG for 1 h. The cultures were serially diluted 5-fold, spotted on YPD plates, and incubated for 2 days at 30 °C. (*F*) WT, *ubr1*Δ and double-mutant *ubr1*Δ *ufd4*Δ *S. cerevisiae* that expressed chromosomally integrated $MGT1_{m13}$ (see *SI Materials and Methods*) were grown at 30 °C to A_{600} of ≈0.8, and were further incubated for 1 h at 37 °C, followed by a ''chase'' with cycloheximide, also at 37 °C. SDS/4–12% NuPAGE of cell extracts was followed by IB with anti-myc and anti-tubulin antibodies. (*G* and *H*) WT and *ufd4*Δ *S. cerevisiae* with chromosomally integrated $MGT1_{m13}$ (see *SI Materials and Methods*) were grown in SC medium at 30 °C to A_{600} of ≈0.8 and were further incubated in the presence of MNNG (68 μM) for 1 h (panel *G*), or at 37 °C (panel *H*), followed by a ''chase'' with cycloheximide for 10, 20, and 40 min, fractionation of cell extracts by SDS/4–12% NuPAGE, and IB with anti-myc and anti-tubulin antibodies.

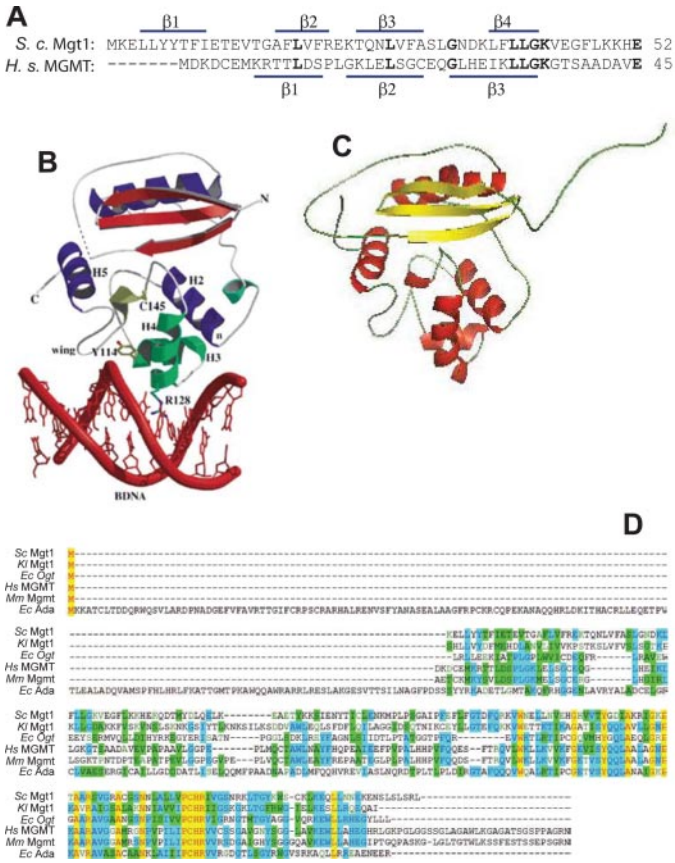

Fig. S3. (*A*) Four putative β-sheets in the N-terminal region of *S. cerevisiae* Mgt1 that are sequelogous (similar in sequence), and probably also spalogous (spatially similar) (1) to the corresponding regions of human Mgmt (MGMT), the structure of which was determined previously (2). Residues identical between *S. cerevisiae* Mgt1 and human MGMT are in bold. (*B*) The structure of human Mgmt and a model of its interaction with DNA (2). Alkyl lesions are removed by Mgt1 and Mgmt from DNA through a base-flipping mechanism (3). (*C*) The hypothetical 3D structure of *S. cerevisiae* Mgt1, produced by Modeller-9v1 (http://salilab.org/modeller/), using the structure of human Mgmt (1eh6.pdb) as its template, and displayed using PyMol (DeLano Scientific). (*D*) Sequence alignments among O^6-alkyguanine transferases of the yeasts (fungi) *S. cerevisiae* (Sc) and *Kluyveromyces lactis* (Kl), the prokaryote *E. coli* (Ec), and the mammals *Homo sapiens* (Hs) and *Mus musculus* (Mm).

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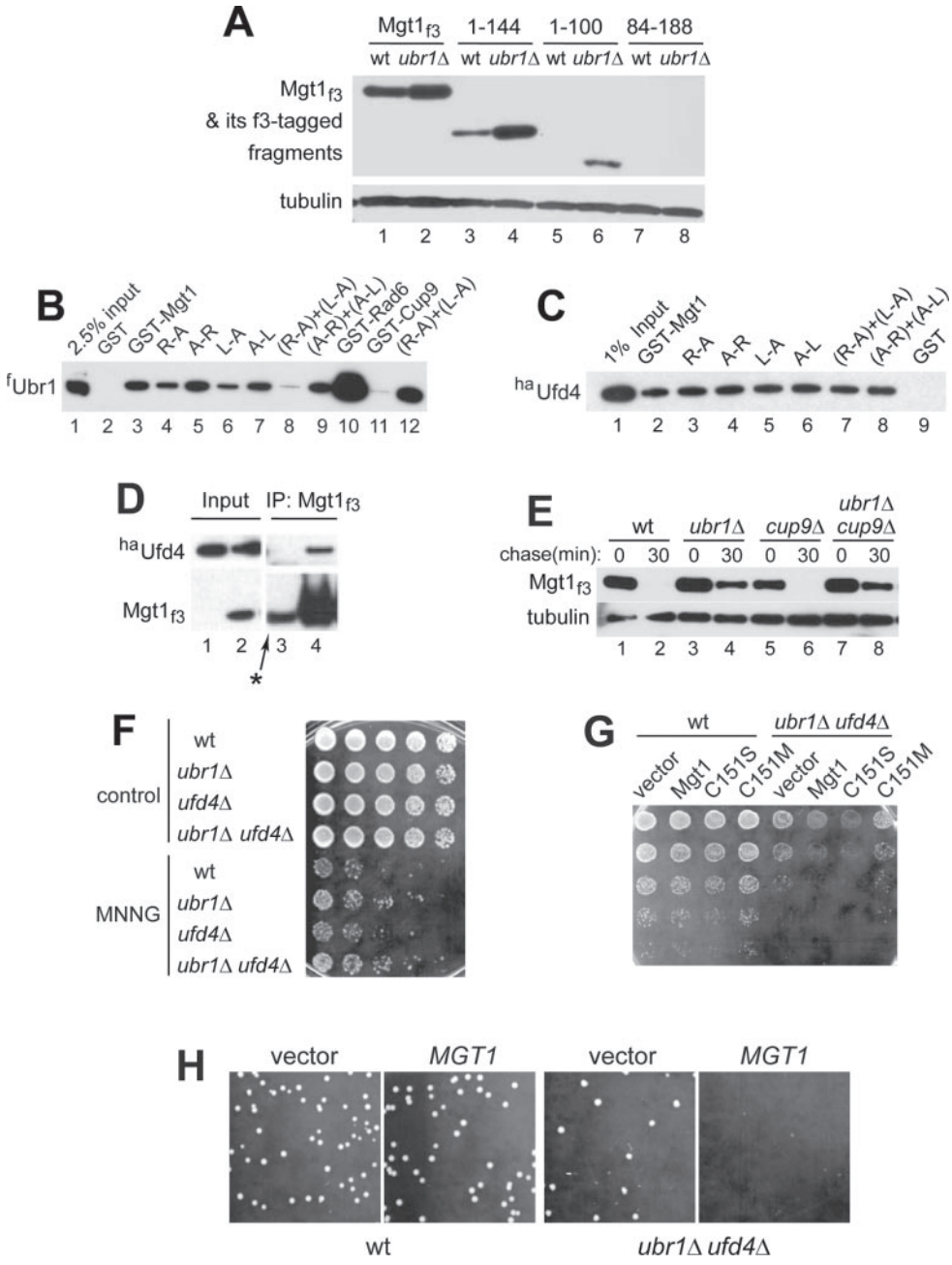

Fig. S4. (*A*) JD52 (WT) and JD55 (*ubr1*Δ) *S. cerevisiae* expressing either full-length $Mgt1_{f3}$ (from the P_{MGT1} promoter on a low copy plasmid) or its C-terminally truncated derivatives were grown to A_{600} of ≈0.8, followed by SDS/4–12% NuPAGE of cell extracts and IB with anti-flag and anti-tubulin antibodies. Lanes 1 and 2, full-length $Mgt1^{1-188,f3}$ in WT and *ubr1*Δ cells, respectively. Lanes 3 and 4, same as lanes 1 and 2, respectively, but with $Mgt1^{1-144,f3}$. Lanes 5 and 6, same but with $Mgt1^{1-100,f3}$. Lanes 7 and 8, same but with $Mgt1^{84-188,f3}$. (*B*) Equal amounts of extracts (50 μg) from *S. cerevisiae* that expressed the N-terminally flag-tagged Ubr1 (^{f}Ubr1) were incubated in either the presence or absence of specific dipeptides (each of them at 1 mM) with GST alone or specific GST fusions (≈5 μg) that were linked to glutathione-Sepharose beads. Bound proteins were eluted from the beads and fractionated by SDS/4–12% NuPAGE, followed by IB with anti-flag antibody. Lane 1, 2.5% of the initial (total) input of ^{f}Ubr1. Lane 2, GST alone. Lane 3, GST-Mgt1. Lane 4, same as lane 3 but with Arg-Ala (R-A, 1 mM). Lane 5, same as lane 4 but with Ala-Arg (A-R, 1 mM). Lane 6, same as lane 3 but with Leu-Ala (L-A, 1 mM). Lane 7, same as lane 3 but with Ala-Leu (A-L, 1 mM). Lane 8, same as lane 3 but with both Arg-Ala and Leu-Ala (R-A and L-A, 1 mM each). Lane 9, same as lane 3 but with both Ala-Arg and Ala-Leu (A-R, A-L, 1 mM each). Lane 10, GST-Rad6. Lane 11, GST-Cup9. Lane 12, same as lane 11 but with both Arg-Ala and Leu-Ala (R-A and L-A, 1 mM each). (*C*) Equal amounts of extracts (0.2 mg) from *S. cerevisiae* that expressed the N-terminally ha-tagged Ufd4 (^{ha}Ufd4) were incubated with GST alone or specific GST fusions (≈5 μg), and were processed as described in *B*, except that IB that ^{ha}Ufd4 was detected by was performed with anti-ha antibody. Lane 1, 1% of the initial (total) input of ^{ha}Ufd4. Lane 2, GST-Mgt1. Lane 3, same as lane 2 but with Arg-Ala (R-A, 1 mM). Lane 4, same as lane 2 but with Ala-Arg (A-R, 1 mM). Lane 5, same as lane 2 but with Leu-Ala (L-A, 1 mM). Lane 6, same as lane 2 but with Ala-Leu (A-L, 1 mM). Lane 7, same as lane 2 but with both Arg-Ala and Leu-Ala (R-A and L-A 1 mM each). Lane 8, same as lane 2 but with both Ala-Arg and Ala-Leu (A-R and A-L, 1 mM each). Lane 9, GST alone. (*D*) Mgt1 interacts with Ufd4. Extracts (1 mg) from *S. cerevisiae* cells coexpressing ^{ha}Ufd4 (from the pRS313CUP1haUFD4 plasmid; Table S2) and either a vector alone or $Mgt1_{f3}$ (from the plasmid pCH437) were precipitated with anti-flag antibody, using protein G linked to magnetic beads. Immunoprecipitates were fractionated by SDS/4–12% NuPAGE, followed by IB with anti-ha and anti-flag antibodies. Lane 1, input sample (0.25% of extract used in immunoprecipitation assays) from cells expressing ^{ha}Ufd4 and the (''empty'') vector p416*MET25*. Lane 2, same as lane 1 but from cells coexpressing ^{ha}Ufd4 and $Mgt1_{f3}$. Lane 3, ^{ha}Ufd4, immunoprecipitation with anti-flag antibody in the absence of coexpressed $Mgt1_{3f}$. Lane 4, same as lane 3 but ^{ha}Ufd4 was coexpressed with $Mgt1_{f3}$. The band detected in lane 3 with anti-flag antibody and marked by an arrow and asterisk, just below the position of $Mgt1_{f3}$ (see lane 4), is the light chain of anti-flag antibody that was used for immunoprecipitation as well (see above). Because of light-chain's abundance at that position on the IB membrane, this otherwise weak cross-reaction was detectable at the level of IB exposure shown (compare the level of the cross-reacting band with the levels of immunoprecipitated Mgt1 in lane 4). (*E*) Cup9-regulated circuits do not influence the Ubr1-mediated degradation of Mgt1. AVY16 (WT), AVY4 (*ubr1*Δ), AVY50 (*cup9*Δ), or AVY51 (*ubr1*Δ *cup9*Δ) *S. cerevisiae* (Table S2) expressing $Mgt1_{f3}$ from the P_{MGT1} promoter on a low copy plasmid were grown to A_{600} of ≈0.8 in SC(-Ura) medium at 30 °C, then incubated further at 37 °C for 1 h, followed by a 30-min ''chase'' with cycloheximide (at 50 μg/ml), fractionation of cell extracts by SDS/4–12% NuPAGE and IB with anti-flag and anti-tubulin antibodies. Note that $Mgt1_{f3}$ remained short-lived in *cup9*Δ cells (lanes 5, 6). (*F*) JD52 (WT), JD55 (*ubr1*Δ), CHY194 (*ufd4*Δ), or CHY195 (*ubr1*Δ *ufd4*Δ) *S. cerevisiae* (Table S1) were grown to A_{600} of ≈0.6, and treated with MNNG (170 μM) for 1.5 h at 30 °C. The resulting cultures were serially diluted 5-fold, spotted on YPD plates, and incubated for 2 days at 30 °C. See also the main-text Fig. 3H. (*G*) A double-mutant *ubr1*Δ *ufd4*Δ strain of *S. cerevisiae* is hypersensitive to overexpression of Mgt1. JD52 (WT) and CHY195 (*ubr1*Δ *ufd4*Δ) cells carrying a vector alone (pRS426*MET25*), or expressing either $Mgt1_{f3}$ (pCH440), or $Mgt1^{C151S,f3}$ (pCH441), or $Mgt1^{C151M,f3}$ (pCH442) were grown in SD medium (with essential amino acids) to A_{600} of ≈0.8, serially diluted, and spotted on SD medium for 2 days at 30 °C. (*H*) Same as in *C* but single-colony growth assays with JD52 (WT) and CHY195 (*ubr1*Δ *ufd4*Δ) cells carrying either a vector alone or expressing $Mgt1_{f3}$ from the P_{MET25} promoter on a high copy plasmid.

Table S1. Some of the *S. cerevisiae* strains used in this study

Strains	Genotypes and characteristics	Source or ref.
JD52	*MATa trp1-Δ 63 ura3–52 his3-Δ 200 leu2–3112. lys2–801*	1
JD55	*ubr1Δ::HIS3* in JD52	1
SC295	*MATa ura3–52 leu2–3,112 regl-501 gal1 pep4–3*	2
AVY4	*ubr1Δ::LEU2* in JD52	Laboratory collection
AVY16	*leu2–3,112::LEU2* in JD52	Laboratory collection
AVY50	*cup9Δ::LEU2* in JD52	Laboratory collection
AVY51	*cup9Δ::LEU2* in JD55	Laboratory collection
AVY105	*ubr2Δ::HIS3* in JD52	Laboratory collection
CHY49	*pdr5Δ::KanMX6* in JD52	3
CHY50	*pdr5Δ::KanMX6* in JD55	3
CHY92	*GAL80::3HA::KanMX6* in JD52	This study
CHY93	*GAL80::3HA::KanMX6* in JD52	This study
CHY94	*KnaMX6::*P_{GAL1}*::3HA-SRS2*	This study
CHY95	*KnaMX6::*P_{GAL1}*::3HA-SRS2*	This study
CHY101	*SGS1::13MYC::TRP1* in JD52	This study
CHY102	*SGS::13MYC::TRP1* in JD55	This study
CHY107	*MGT1::13MYC::TRP1* in JD52	This study
CHY108	*MGT1::13MYC::TRP1* in JD55	This study
CHY109	*CCR4::3HA::TRP1* in JD52	This study
CHY110	*CCR4::3HA::TRP1* in JD55	This study
CHY115	*MGT1::13MYC::TRP1 pdr5Δ::KanMX6* in JD52	This study
CHY116	*MGT1::13MYC::TRP1 pdr5Δ::KanMX6* in JD55	This study
CHY121	*mgt1Δ::KanMX6* in JD52	This study
CHY122	*mgt1Δ::KanMX6* in JD55	This study
CHY134	*ubr1Δ::LEU2 ubr2Δ::HIS3* in JD52	This study
CHY161	*PEX7::3HA* in JD52	This study
CHY162	*PEX7::3HA::TRP1* in JD55	This study
CHY163	*YGL081W::13MYC::TRP1* in JD52	This study
CHY164	*YGL081W::13MYC::TRP1* in JD55	This study
CHY169	*MGT1::13MYC::TRP1 ufd4Δ::KanMX6* in JD55	This study
CHY107	*MGT1::13MYC::TRP1* in JD52	This study
CHY108	*MGT1::13MCY::TRP1* in JD55	This study
CHY194	*ufd4Δ::KanMX6* in JD52	This study
CHY195	*ubr1Δ::HIS3 ufd4Δ::KanMX6* in JD52	This study
CHY219	*ufd4Δ:: TRP1 MGT1–13MYC::KanMX6* in JD52	This study
YPH277	*MATa ura3–52 ade2–101 trp1-D1 leu2-D1 CFVII (RAD2.d) URA3 SUP11*	4
YPH277HR1	*ubr1Δ::LEU2* in YPH277	5
BY4425	*ubc2Δ:: KanMX4* in BY4741	Open Biosystems
BY3216	*ubc4Δ:: KanMX4* in BY4741	Open Biosystems
BY3994	*ubc5Δ::KanMX4* in BY4741	Open Biosystems
BBY67.3	*ubc6Δ::HIS3* in JD52	Laboratory collection
BY597	*ubc7Δ::KanMX4* in BY4741	Open Biosystems
BY6577	*ubc8Δ:: KanMX4* in BY4741	Open Biosystems
BY4763	*ubc10Δ:: KanMX4* in BY4741	Open Biosystems
BY1636	*ubc11Δ:: KanMX4* in BY4741	Open Biosystems
BY5214	*ubc12Δ:: KanMX4* in BY4741	Open Biosystems
BY4027	*ubc13Δ:: KanMX4* in BY4741	Open Biosystems
BY4454	*mms2Δ:: KanMX4* in BY4741	Open Biosystems
BY3771	*bre1Δ:: KanMX4* in BY4741	Open Biosystems
BY6430	*rad5Δ:: KanMX4* in BY4741	Open Biosystems
BY5787	*rad18Δ:: KanMX4* in BY4741	Open Biosystems
BY4425	*ufd2Δ:: KanMX4* in BY4741	Open Biosystems
BY3216	*ufd4Δ:: KanMX4* in BY4741	Open Biosystems
BY3994	*hul4Δ::KanMX4* in BY4741	Open Biosystems
BY597	*hul5Δ::KanMX4* in BY4741	Open Biosystems
BY6577	*tom1Δ:: KanMX4* in BY4741	Open Biosystems
BY4763	*hrd1Δ:: KanMX4* in BY4741	Open Biosystems
BY4156	*hrd3Δ:: KanMX4* in BY4741	Open Biosystems
BY4883	*tul1Δ:: KanMX4* in BY4741	Open Biosystems
BY7299	*doa10Δ::KanMX4* in BY4741	Open Biosystems
BY4814	*ubr1Δ:: KanMX4* in BY4741	Open Biosystems
BY1579	*ubr2Δ:: KanMX4* in BY4741	Open Biosystems
BY3771	*bre1Δ:: KanMX4* in BY4741	Open Biosystems
BY6430	*rad5Δ:: KanMX4* in BY4741	Open Biosystems
BY5787	*rad18Δ:: KanMX4* in BY4741	Open Biosystems
MGG15	*ura3–52 cdc34–2*	6
FW1808	*MAT Δhis4–912 R5 Δlys2–128 Δura3–52 rps5–1*	7

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6. Goebl MG, *et al.* (1988) The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science* 241:1331–1335.
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Table S2. Some of the plasmids used in this study

Plasmids	Characteristics	Source or ref.
pFlagUBR1SBX	^{f}Ubr1 in YEplac181, with P_{ADH1} promoter	1
pMET416$_{F}$UPRCUP9$_{NSF}$	^{f}DHFR-Ub^{K48R}-Cup9$_{NSF}$ in pRS416MET25	2
P313CUPHaUFD4	pRS313CUP1-^{ha}UFD4	3
p314CUP1FlagUFD4	pRS314CUP1-^{f}UFD4	Laboratory collection
pUB203	pRS424CUP1-UBI^{K48R,G76A}	Laboratory collection
pUB223	pRS424CUP1-His6-MYC-UBI^{K48R,G76A}	Laboratory collection
pCH100	pRS315-UBR1	4
pCH159	pRS315-UBR1^{C1220S}	4
pCH279	pRS416MET25-MGT1$_{ha}$	This study
pCH280	pRS416MET25-^{f}DHFR-Ub^{K48R}-MGT1^{f}	This study
pCH281	pRS426MET25-^{f}DHFR-Ub^{K48R}-MGT1^{f}	This study
pCH285	pGEX4T-3-MGT1$_{his10}$	This study
pCH336	pRS416-MGT1$_{3f}$	This study
pCH337	pRS416-MGT1^{C151S,3f}	This study
pCH338	pRS416-MGT1^{C151M,3f}	This study
pCH371	pRS416-MGT1$^{84–188,3f}$	This study
pCH372	pRS416-MGT1$^{1–100,3f}$	This study
pCH374	pRS416-MGT1$^{1–144,3f}$	This study
pCH437	pRS416MET25-MGT1$_{3f}$	This study
pCH438	pRS416MET25-MGT1^{C151S,3f}	This study
pCH439	pRS416MET25-MGT1^{C151M,3f}	This study
pCH440	pRS426MET25-MGT1$_{3f}$	This study
pCH441	pRS426MET25-MGT1^{C151S,3f}	This study
pCH442	pRS426MET25-MGT1^{C151M,3f}	This study

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4. Hwang C-S, Varshavsky A (2008) Regulation of peptide import through phosphorylation of Ubr1, the ubiquitin ligase of the N-end rule pathway. *Proc Natl Acad Sci USA* 105:19188–19193.

Table S3. Some of the PCR primers used in this study

Name	Sequence
OCH450	AAGATTACCAACGCCAGCCATATT
OCH492	ACAGGATCCATGAAGGAACTGCTTTACTATACA
OCH494	GATCCCGCGGTGGT ATGAAGGAACTGCTTTACTATACA
OCH495	AACGAATTCTTACTTGTCATCATCGTCCTTGTAGTCGGAAGTACCAGACAATCTACTAAGGCTTAAGCTATT
OCH498	AAACTCGAGCTAGTGATGATGGTGATGATGGTGATGGTGATGAGAACCTCTCAATCTACTAAGGCTTAAGCTATT
OCH540	AACCTGGCATTGTTAGTACCTTCTCATAGAATCGTTGGTAGCAAT
OCH541	ATTGCTACCAACGAT TCTATGAGAAGGTACTAACAATGCCAGGTT
OCH553	AACCTGGCATTGTTAGTACCTATGCATAGAATCGTTGGTAGCAAT
OCH554	ATTGCTACCAACGAT TCTATGCATAGGTACTAACAATGCCAGGTT
OCH576	AAC GGATCC CCTATAGAAA CCCGTGACGA AAGG
OCH577	AAACTCGAGTTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACCGTCATGGTCTTTATAGTCACTAGTCAATCTACTAAGGCTTAAGCTATT′